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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/53, C07K 16/00	A1	(11) International Publication Number: WO 99/45393 (43) International Publication Date: 10 September 1999 (10.09.99)
(21) International Application Number: PCT/US99/04955 (22) International Filing Date: 5 March 1999 (05.03.99) (30) Priority Data: 09/035,708 5 March 1998 (05.03.98) US (71) Applicant: UNIVERSITY OF CINCINNATI [US/US]; 231 Bethesda Avenue, Cincinnati, OH 45267 (US). (72) Inventors: ZEMLAN, Frank, P.; 7000 Drake Road, Cincinnati, OH 45243 (US). CAMPBELL, Thomas, A.; 9833 Hocking Street, Massillon, OH 44646 (US). (74) Agent: GILCREST, Roger, A.; Standley & Gilcrest, Suite 210, 495 Metro Place South, Dublin, OH 43017 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: METHOD OF DETECTING AXONAL DAMAGE, ASSOCIATED DISEASE STATES, AND RELATED MONOCLONAL ANTIBODIES AND PROTEIN CONTROLS THEREFOR (57) Abstract <p>Patients having several neurological diseases have been shown to have elevated levels of axonally-derived proteins (i.e. tau and neurofilament proteins) in cerebrospinal fluid (CSF) and in brain tissue. Three monoclonal antibodies (MAbs) recognizing CSF tau proteins were developed. The MAbs were found to label a ladder of 30 kD to 50 kD tau proteins in CSF from patients with disease states producing axonal damage such as head trauma or CNS tumor but not in CSF from controls. High levels of tau protein in CSF were shown to be diagnostic of axonal degeneration in head trauma. An ELISA assay was developed with these MAbs to aid in the diagnosis of patients with axonal damage.</p>		

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Method of Detecting Axonal Damage, Associated Disease States, and Related Monoclonal Antibodies and Protein Controls Therefor

Field of the Invention

The present invention is in the field of clinical and diagnostic testing, and relates generally to a method of detecting axonal damage and associated disease states.

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Background of the Invention

Axonal degeneration is a primary feature of brain injury in humans (Hayes et al., 1995). The present invention describes several methods of assessing axon degeneration in humans by measuring proteins that are localized in axons. These axonal proteins are released following head injury into the extracellular space and are transported to cerebrospinal fluid (CSF). Methods are disclosed in the present invention for measuring these axonal proteins in the CSF of patients.

Tau protein is a major microtubule associated structural protein localized primarily in axons (Binder et al. 1985; Kosik and Finch 1987). The localization of tau in axons is thought to result from the preferential sequestration of tau mRNA in the proximal portion of axons (Litman et al., 1993) and the selective stabilization of tau in axons (Kanai and Hirakawa 1995). Human tau proteins are encoded by a single gene and at least six alternately spliced isoforms have been identified that demonstrate an apparent molecular weight of 48 kilodalton (kDa) to 68 kDa (Goedert et al. 1989 and Figure 5). Under normal conditions, little or no tau is released extracellularly. This disclosure teaches that tau is released under clinical conditions associated with axon damage.

Neurofilament proteins, similar to tau, are structural neuronal proteins found in central nervous system axons (Shaw, 1986). Neurofilaments that are the subject of the present invention

consist of four separate protein elements: 1) a light neurofilament subunit (neurofilament-L) with an apparent molecular weight of 68 kDa, 2) a medium-sized neurofilament subunit (neurofilament-M) with an apparent molecular weight of 160 kDa, 3) a heavy neurofilament subunit (neurofilament-H) with an apparent molecular weight of 200 kDa, and 4)

5 neurofilament66/ α -internexin (neurofilament66) with an apparent molecular weight of 66 kDa (Lee and Cleveland, 1996). These neurofilaments are each encoded on a separate gene (Julien et al., 1987; Myers et al., 1987; Lees et al., 1988; Chan and Chiu, 1995). Following head injury neurofilaments are depleted from degenerating axons and gain access to the CSF (Hayes et al., 1995).

10 One clinical condition associated with axonal degeneration is head trauma. Axonal injury, clinically referred to as diffuse axonal injury, accounts for about half of the primary lesions observed in closed head trauma and is one of the most frequent causes of poor clinical outcome. MRI is the procedure of choice for detecting diffuse axonal injury, however, MRI routinely underestimates the true extent of the damage. One embodiment of the present invention
15 involves the development and use of an alternative procedure for quantifying axon damage in patients with CNS injury.

Another embodiment of the present invention involves a method for quantifying axonal degeneration which is the central feature of neurodegenerative disorders including Alzheimer's disease. Alzheimer's disease is a progressive, degenerative disease that attacks the brain and
20 results in impaired memory, thinking and behavior. Alzheimer's disease is the most common form of dementia, which is the loss of intellectual function so severe it interferes with daily life. Since being first described by Dr. Alois Alzheimer in 1906, it has become the fourth leading cause of death among adults in the United States between the ages of 75 and 84. The clinical

mortality and morbidity seen in Alzheimer's patients directly results from neuronal death in the brain. In neurodegenerative disorders including Alzheimer's disease, neuronal death is always accompanied by axonal degeneration.

To determine the existence *vel non* of disease or trauma states associated with axonal
5 damage in a patient, it is desirable to be able to ascertain whether a patient has such axonal damage and to quantify such damage.

Accordingly, it is desirable to be able to perform convenient and reliable tests for tau proteins or neurofilament proteins in order to test for axonal damage. It is also desirable to have available such tests that may be used on *ante mortem* or *post mortem* patient samples.

10 In view of the present disclosure or through practice of the present invention, other advantages or problem solutions may become apparent.

Summary of the Invention

The present invention includes a method of determining the extent of axonal damage in
15 the human CNS, novel cleaved forms of tau proteins and neurofilament proteins associated with axonal damage, and monoclonal antibodies (Mabs) raised against novel cleaved forms of tau protein useful in such a method.

In general terms, the method of the present invention is a method of determining axonal damage in the human CNS, the method comprising the steps: (a) obtaining a sample of CSF from
20 the human central nervous system of a patient; (b) treating the sample of CSF with monoclonal antibody(ies) binding to novel cleaved form(s) of tau proteins or neurofilament proteins described in the present application; and (c) detecting the presence and/or level of the cleaved form(s) of the tau proteins or neurofilament proteins bound to the monoclonal antibody(ies).

The method of the present invention may also include the step of comparing the amount of the cleaved form(s) of the tau proteins or neurofilament proteins bound to the monoclonal antibody(ies) in step (c) to control samples selected from the group consisting of those representing a normal undamaged axon state and those representing an axonal damage state.

5 The method of the present invention may be used to detect cleaved form(s) of tau proteins or neurofilament proteins. The cleaved tau protein(s) of particular interest in the present method are those having an apparent molecular weight less than 50 kDa and being in a phosphorylated or non-phosphorylated state, particularly those fragments in the range of about 30 kDa to 50 kDa. The present method also has been forced to characterize the multiple protein bands comprising
10 these 30 kDa to 50 kDa tau protein fragments. The cleaved neurofilament proteins of particular interest in the present method occur in human CSF where they demonstrate an apparent molecular weight: 1) for neurofilament-L less than 68 kDa, 2) for neurofilament-M less than 160 kDa, 3) for neurofilament-H less than 200 kDa, and for neurofilament66 less than 66 kDa.

The method of the present invention may be used to determine whether axonal damage
15 has occurred and to what extent, and thus can be used to determine the existence or likelihood of any disease state associated with axonal damage, such as CNS injuries, including primary neuronal injuries (e.g., cortical contusion, diffuse axonal injury, subcortical gray matter injury and primary brain stem injury), primary hemorrhages (e.g., subdural hematoma, epidural hematomas, intracerebral hematoma and diffuse hemorrhages), primary vascular injuries (e.g.,
20 arterial psuedoaneurysm, arterial dissection/occlusion), dural sinus laceration/occlusion, traumatic pia-arachnoid injuries, cranial nerve injuries and secondary traumatic lesions (e.g., infarction, hypoxic injury, diffuse brain swelling/edema, secondary hemorrhage), central nervous system tumor, neurodegenerative diseases of the central nervous system including Alzheimer's

disease, spinal cord injury, acute cerebral vascular accident or axonal damage following ingestion of drug(s) or poison(s).

The present invention also includes cleaved forms of tau proteins having an apparent molecular weight less than 50 kDa particularly those fragments in the range of about 30 kDa to 50 kDa, and being in a substantially isolated and/or purified form. The present invention also includes cleaved forms of neurofilament proteins in a substantially isolated form occurring in human CSF where they demonstrate an apparent molecular weight: 1) for neurofilament-L less than 68 kDa, 2) for neurofilament-M less than 160 kDa, 3) for neurofilament-H less than 200 kDa, and for neurofilament66 less than 66 kDa. These substances may be used as standards or controls in tests using the present invention.

Substantially isolated and/or purified Mabs raised against at least one tau protein and/or its cleaved forms, and having been screened against human CSF are also included in the present invention. As used herein, a substantially isolated or substantially purified monoclonal antibody consists of a single hybridoma clone recognizing a single protein epitope essentially free of contaminating or interfering molecules. It is preferred that such Mabs are those raised against tau protein fragments, and having been screened against human CSF, said monoclonal antibody being in a substantially isolated form, and said tau protein fragment comprising the peptide sequence including the amino acids from serine¹⁹⁹ to serine³⁹⁶ of tau protein, and lacking the native N-terminal and C-terminal amino acids. These substances are useful in the methods of the present invention.

The present invention can also be used to ascertain or predict the severity of neurologic trauma, such as intercranial lesions, or the neurologic disease states giving rise to tau or neurofilament proteins in CSF, such as those disease states discussed above and/or to ascertain or

predict clinical outcome following such trauma.

Description of Figures

Fig. 1. Monoclonal antibodies recognizing cerebrospinal fluid cleaved tau proteins were developed by differential CSF screening.¹²⁴ *Left panel:* Initial studies demonstrated that Mab Tau-1 labeled 30 kD to 50 kD CSF proteins in CNS trauma patients (TRA; lane a 50 μ l CSF, lane b 10 μ l CSF) but not normal controls (CNT; lane c and d both 50 μ l CSF). *Right panel:* Mabs were developed that specifically recognized 30 kD to 50 kD CSF tau proteins employing a differential CSF screen. Hybridomas were selected that labeled CSF from CNS trauma patients (lanes a-d) but not control patients (lanes e-h). The same patient samples were run in lanes a and b in left and right panels. cTau7 lanes a and e-h 50 μ l CSF, lanes b-d 10 μ l CSF. Molecular weight markers shown at right.

Fig. 2. CSF cleaved tau Mabs label recombinant tau. Commassie Blue stained 10% gel with recombinant 3-repeat tau (Gel, left lane, 1 μ g) and 4-repeat tau (Gel, right lane, 1 μ g). Recombinant tau blotted with BYA (0.07 μ g/lane) and cTau12 (2.5 μ g/lane). Molecular weight markers shown at right.

Fig. 3. CSF cleaved tau proteins bind microtubules. *Microtubule Purified:* Initially, taxol polymerized microtubules were salt extracted to insure that no cTau7 immunoreactive proteins were present (lane a). Microtubules were then incubated with a preparation of CSF cleaved tau proteins (lane b, 1 μ g) and washed several times until the supernatant was free of cTau7 reactivity (lanes c and d). Microtubule bound proteins were salt extracted yielding 30 kD to 50 kD cTau7-reactive cleaved tau proteins (lane e). Similar results were obtained with Mabs cTau8 and cTau12 (data not shown). *Affinity Purified:* CSF cleaved tau proteins were affinity purified with

Mabs cTau7, cTau8 and cTau12 coupled to Protein G agarose. Commassie Blue stained gels indicated that affinity purified cleaved tau consisted of a primary 50 kD protein band (gel, 1 µg). Immunoblots with cTau7 revealed a single protein band when 10 nanograms (ng) of affinity purified cleaved tau protein was loaded: lower molecular weight cTau7 reactive proteins were detected with larger protein loads (30 and 100 ng). Similar results were observed with Mabs cTau8 and cTau12 (data not shown).

Fig. 4. Mabs cTau7, cTau8 and cTau12 recognize a 14.7 kDa CNBr cleaved tau digestion fragment occurring in patient CSF or Brain. Cleaved tau from CSF or brain (2 µg/lane) was treated either with (+) or without (0) CNBr and blotted with Mabs PHF-1, cTau7, cTau8 and cTau12. All four antibodies predominantly labeled the same CNBr digestion fragment in both CSF and brain consisting of pro²⁵¹ to met⁴¹⁹ of tau. The PHF-1 fragment has a reported molecular weight of 14.7 kDa (Zemlan and Dean, 1996). Blots are from a 15% gel. The position at which non-digested cleaved tau migrated is shown (Tau).

Fig. 5. CNS trauma brain contains both full length 48 kD to 68 kD tau proteins and 30 kD to 50 kD cleaved tau proteins. Full length brain tau proteins were selectively labeled with the tau antibody BYA, the C-terminal tau antibody Alz5 and the N-terminal tau antibody Alz50. Cleaved tau proteins were selectively labeled with Mabs cTau7, cTau8, cTau12 and PHF1 that recognizes phospho-ser³⁹⁶ of tau. Mab Tau-1 that recognizes non-phosphorylated ser¹⁹⁹ of tau labeled both forms of tau. These data suggest that tau is cleaved and phosphorylated at ser¹⁹⁹ in brain.

Fig. 6. Titration of affinity purified CSF cleaved tau employing the developed cleaved tau ELISA. A catalyzed-reporter deposition sandwich ELISA was developed employing cTau12 as capture antibody and HRP-conjugated cTau7 and cTau8 for detection. Affinity purified CSF cleaved tau was used as standard. All concentrations (pg/well) were tested in triplicate and values

represent the mean O.D. value.

Detailed Description of Invention

The proteins that are useful in the present invention are a novel class of axonally-derived
5 proteins occurring in human CSF including cleaved tau proteins and cleaved neurofilament
proteins. As used herein, axonally-derived proteins refer to a group of proteins that occur in
axons of CNS neurons and are released into the extracellular space during degeneration of said
axons. As used herein, the term full length tau refers to any of the six non-cleaved isoforms of
10 and amino acid sequences are described in Goedert et al. (1989). The class of novel cleaved tau
proteins that are useful in the invention were discovered in human cerebrospinal fluid and have
been purified. These novel cleaved tau proteins may be distinguished from previously described
full length tau proteins, in that they: 1) have reduced apparent molecular weights in comparison
to full length tau, 2) are comprised of the interior portion of the tau sequence that includes ser¹⁹⁹
15 to ser³⁹⁶ of tau, and 3) lack the N-terminal and C-terminal amino acids of tau. As used herein, the
term neurofilament-L refers to a protein with an apparent molecular weight of 68 kDa and whose
genomic and amino acid sequences are described in Julien et al. (1987). As used herein, the term
neurofilament-M refers to a protein with an apparent molecular weight of 160 kDa and whose
genomic and amino acid sequences are described in Myers et al. (1987). As used herein, the term
20 neurofilament-H refers to a protein with an apparent molecular weight of 200 kDa and whose
genomic and amino acid sequences are described in Lees et al. (1988). As used herein, the term
neurofilament66 refers to a protein with an apparent molecular weight of 66 kDa and whose
genomic and amino acid sequences are described in Chan and Chiu (1995).

Antibodies specifically reactive with the novel cleaved tau proteins are included within the scope of the invention. A "specifically reactive" antibody is one that is capable of binding with a particular molecule to thereby couple said molecule to the antibody. The term "epitope" refers to that portion of a hapten which can be recognized and bound by an antibody. The present disclosure indicates that the antigen employed for monoclonal antibody production, a cleaved form of tau found in human cerebrospinal fluid, possesses more than one epitope. An antigen is a molecule capable of inducing an animal to produce an antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that an antibody will bind with a significantly higher affinity to its corresponding antigen as opposed to the multitude of other antigens occurring in the human body. For example, there is provided by this invention several Mabs (i.e. cTau-7, cTau-8 and cTau-12) raised against the cleaved form of tau found in CSF. These cTau antibodies demonstrated sufficiently higher affinity for cleaved tau than for full length tau such that selective immunolabeling of cleaved tau occurred with equivalent protein loads on Western blots.

15

Example:

Purification, Characterization and Measurement of Human Cerebrospinal Fluid Cleaved Tau Proteins in Patients with Axonal Degeneration

Summary of Example

Tau proteins are structural axonal proteins associated with microtubule stabilization. Under normal conditions tau is a non-released intraneuronal protein, however, the disclosure teaches that tau gains access to the CSF during axon degeneration. As described in the present disclosure, the cleaved form of tau proteins found in human CSF during axon degeneration were purified, characterized and Mabs that selectively bind to this novel cleaved form of tau

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developed. These monoclonals were employed to develop a sandwich ELISA for measuring CSF cleaved tau. Employing this ELISA, patients with axonal degeneration demonstrated CSF cleaved tau levels 10,000 times higher than patients without axonal degeneration; no overlap in CSF cleaved tau levels between the two groups of patients was observed.

5

Materials and Methods

CSF samples

CSF samples were collected under an approved protocol from the University of Cincinnati Institutional Review Board. CSF was collected from hospitalized patients with
10 severe brain injury (Glasgow Coma Scale < 10) resulting from trauma or intracranial aneurysmal rupture via intraventricular catheters. CSF was collected by lumbar puncture from control patients. CSF samples were centrifuged at 13,000 g for 15 min and stored at -70°C until use.

15 Protein purification and expression

Tau purification, dephosphorylation and digestion. Tau was purified using a procedure modified from Nukina et al. (1987). Briefly, post mortem human brains were homogenized in a volume 2.5 times the weight in 50 mM Tris-HCl (pH 6.8), 0.3 M NaCl, 1% β -mercaptoethanol (BME), 1 mM PMSF and 5 μ M leupeptin. The homogenate was centrifuged
20 at 30,000 x g for 5 min at 4°C. The supernatant was incubated on ice and the pellet homogenized a second time in a volume 2.5 times the weight with 50 mM Tris-HCl (pH 9.2), 0.3 M NaCl, 1% BME. The homogenate was then centrifuged at 4°C for 5 min at 30,000 x g. The supernatants were combined and boiled for 10 min. The samples were then spun for 30

minutes at 30,000 x g at 4°C. The samples were dialyzed overnight against 50 mM Tris-HCl before examination by SDS-PAGE. Tau antigen was prepared by running the tau preparation on 10% SDS-polyacrylamide curtain gels. Proteins with molecular weights of 30 kDa to 80 kDa were excised and electroeluted in a Schleicher & Schuell (Keene, NH) Elutrap device
5 containing 40 mM Tris-borate buffer (pH 8.64) and 0.8% SDS, then dialyzed against 50 mM Tris-HCl prior to injection. Brain and CSF tau proteins were dephosphorylated overnight at 37°C in 50 mM Tris-HCl (pH 8.0) with or without 1 U/ml bacterial alkaline phosphatase (BAP; Sigma Chemical Co.). The effect of BAP treatment on Mab binding was assessed by solid phase ELISA. Wells were coated with 300 ng of tau, blocked and washed, and primary
10 antibody added for 1 hr and assayed as described below. For epitope mapping studies, tau samples were digested in 200 µl of 70% formic acid containing 50 mg/ml cyanogen bromide (CNBr) solution and incubated overnight at room temperature. Samples were washed twice with 1 ml ddH₂O and evaluated on 15% SDS-PAGE gels.

Purification of microtubules. Tubulin was purified from rat brain and microtubules
15 assembled in the presence of taxol (Schiff et al., 1979; Vallee, 1982). Microtubule-associated proteins (MAPs) were dissociated from the microtubules by suspending the microtubule pellet (600 µg) in assembly buffer (40 µM taxol in 0.1 M PIPES, 1.0 mM EGTA, 1.0 M MgSO₄ and 1.0 mM GTP,) and NaCl added to a final concentration of 0.35 M. CSF and brain samples were incubated with shaking for 10 min at 37°C and centrifuged at 30,000 x g for 25
20 min. After repeated salt extractions (3X), all MAPs had been removed from the microtubule samples as judged by Western blots of the third supernatant probed with BYA.

MAP purification. NaCl was removed from the above microtubule pellet (600 µg) by washing in 1.3 ml of assembly buffer. Microtubules were pelleted at 30,000 x g for 25 min;

CSF or human heat-stable proteins containing tau and 1.3 ml assembly buffer were added to the pellet, incubated for 10 min at 37°C, and centrifuged at 30,000 x g for 25 min. Proteins not bound to microtubules were removed by two cycles of washing (1.3 ml assembly buffer, incubation for 10 min at 37°C, centrifugation at 30,000 x g for 25 min). Microtubule bound
5 proteins were dissociated by the addition of 0.5 ml of assembly buffer with 0.35 M NaCl to the washed microtubule pellet. Following incubation for 10 min at 37°C the sample was dialyzed against 50 mM Tris-HCl and analyzed by immunoblotting.

Recombinant tau. Recombinant human tau was produced using the previously described pET-n123c and pET-n1234c plasmids expressed in the BL21(DE3) expression vector
10 (Lee and Rook, 1992). The plasmids were the generous gift of Dr. Gloria Lee. Plasmid pET-n123c codes for the 352 amino acid three repeat form of tau while plasmid pET-n1234c codes for the 383 amino acid four repeat form of tau containing exon 10. Following expression tau was purified as above and was estimated to be approximately 90% pure as judged by Commaassie Blue stained SDS-PAGE.

15

Monoclonal antibody production

Monoclonal human cleaved tau antibodies. Female Balb/C mice were injected interperitoneally with 100 µg of the antigen preparation suspended in an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). Boosts were performed at two week
20 intervals with 100 µg of the antigen suspended in incomplete Freund's adjuvant (Sigma). Mice were bled prior to each boost and sera titered by ELISA. Mabs were produced as previously described (Kohler and Milstein, 1975). Briefly, 1.8×10^8 spleen cells were mixed with 3.6×10^7 NS1/1-Ag-4 mouse myeloma cells. Fusion was induced by addition of 38% polyethylene

glycol 1550. Cells were washed with DMEM (Gibco BRL, Gaithersburg, MD) and resuspended in Super DMEM (Gibco BRL) containing 14% fetal calf serum and HAT (Sigma). Cells were dispersed in 96-well microtiter plates coated with a mouse splenocyte feeder layer. Supernatants were screened after plating against CSF from CNS trauma patients or controls by ELISA and Western blot. Colonies that produced supernatant found to react with CSF from CNS trauma patients but not control CSF were expanded and cloned by limiting dilution. To insure isolation of monoclonal hybridomas, cloning was repeated until 100% of the wells showed specific Mab production. At the end of this process, three Mabs, designated cTau7, cTau8 and cTau12 were recovered.

10 *Ascites production.* Male Balb/C mice were primed by IP injection of 0.5 ml pristane (Sigma) followed 14 days later by injection of 10^6 hybridoma cells. After seven days, the peritoneal cavities were tapped. Ascites fluids were titered, pooled by hybridoma and stored at -20°C . To ensure establishment of stable cell lines, hybridomas were passed twice through pristane primed mice. Following each passage, hybridomas were recloned. Stability was defined as 100% of hybridoma supernatants exhibiting immunoreactivity against antigen by ELISA. Stable hybridomas were then injected into mice and ascites collected for Mab purification (below).

20 *Mab purification and tau affinity purification.* One ml of ascites was diluted 1:1 with 50 mM sodium acetate (pH 5.0) and applied to 2 ml of equilibrated Protein G sepharose. After washing, Mabs were eluted with Gentle Ag/Ab Elution Buffer (Pierce, Rockford, IL) and desalted over sepharose G-25 (2SQ-B, Isolab, Inc., Akron, OH). Mab purity was confirmed by isoelectric focusing using Resolve™ agarose IEF gels (Isolab, Akron, OH) and SDS-PAGE. Purified Mabs 7A5, 8A12 and 12B2 were used to affinity purify human tau using

Protein G-agarose (Boehringer Mannheim, Germany) as specified by the manufacturer. Purified Mabs were conjugated to horseradish peroxidase (HRP; Finnsugar) as described in Boorsma and Kalsbeek (1975). A checkerboard titration was performed to determine the optimal conjugate dilution for ELISA and immunoblot studies.

5

Antibodies

Mabs PHF-1 (IgG, 1:500) and Alz50 (IgM, 1:10) both raised against a PHF preparation (Wolozin et al., 1986; Greenberg et al., 1992) were the generous gift of Dr. Peter Davies. Mab SMI33 (IgM, 1:750) that recognizes non-phosphorylated ser²³⁵ of tau (Lichtenberg-Kraag et al., 1992) was purchased from Sternberger Monoclonals Inc (Baltimore, MD). Polyclonal antibody BYA-1074 (1:1,000) raised against bovine tau (Kosik et al., 1989) and tau Mab Tau-1 (IgG, 1:500) that recognizes non-phosphorylated ser¹⁹⁹ of tau (Liu et al., 1993) were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Polyclonal antibody Alz5 (1:300) was raised against a synthetic peptide corresponding to the C-terminal 13 amino acids of tau (Caputo et al., 1992).

15

Immunoblotting

CSF or non-digested brain proteins were run on 10% SDS-polyacrylamide gels (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (BA-S 85, Schleicher & Schuell, Keene, NH) as described by Towbin (1979). Nonspecific binding was blocked with 5% BSA in TBST (0.1 M Tris-HCl, 0.9% NaCl, with 0.1% (v/v) Tween 20) for 1 hr. The membrane was washed for 30 minutes in TBST, incubated with primary antibody for 1 hr, and blocked in 5% nonfat dry milk in TBST for 15 minutes. After washing (3 times in

20

TBST), a 1:200 dilution of biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) in TBST was added for 30 minutes, the membrane washed and then transferred to Vectastain ABC-peroxidase (Vector Laboratories) in TBST for 30 minutes. The blot was washed and transferred to a substrate solution of diaminobenzidine tetrahydrochloride
5 dehydrate in phosphate-buffered saline (PBS) with 1% NiCl_2 and 1% CoCl_2 . Mabs cTau7, cTau8 and cTau12 were diluted 1:1,000 for immunoblots.

Enzyme-Linked Immunosorbent Assay

Tau sandwich ELISA. Immulon 2 plates were coated with affinity purified Mab cTau12
10 (100 μl /well, 5 $\mu\text{g}/\text{ml}$) for 1 h and overcoated overnight with 5% nonfat dry milk and 0.5% gelatin in TBS. Plates were washed with TBST, 100 μl /well of affinity purified tau was added (27 pg to 1,720 pg), the plates incubated for 1 h and then washed with TBST. A 1:1 mixture of HRP-conjugated Mabs cTau7 and cTau8 (100 μl /well, 1:2,000) was added, and the plates incubated for 1 h and washed with TBST. Biotin-tyramine (100 μl , 3 $\mu\text{g}/\text{ml}$ in 50 mM Tris-
15 HCl, 0.001% H_2O_2 , pH 8.0) was added for 15 min and plates washed (Bobrow et al, 1989). Color was developed with Vector ABC-AP using nitrophenylphosphate as substrate and read at 405 nm. Negative controls included exclusion of cTau12, deletion of tau, or cTau7 and cTau12 from the assay.

20 Results

CSF cleaved tau Monoclonal antibodies

CSF from CNS injury and control patients was immunoblotted with Mab Tau-1 whose epitope has been mapped to ser¹⁹⁹ of tau. Distinct but modest Tau-1 labeling of CSF

proteins in the 30 kDa to 50 kDa region was observed in CNS injury patients while no immunoreactivity occurred in control patients (Fig. 1). Mabs recognizing these 30 kDa to 50 kDa CSF cleaved tau proteins were developed by subjecting hybridoma supernatants to a differential solid phase ELISA screen employing CSF samples from CNS injury and control patients. Three monoclonal producing hybridomas, cTau7, cTau8 and cTau12 were selected that maximally discriminated between CNS injury and control CSF (Fig. 1). For example, cTau7 CSF immunoblot data from four CNS trauma patients are shown in Fig. 1. CSF samples in the first and third lanes were from two different closed head injury patients with intracranial hemorrhage while CSF samples in the second and fourth lanes were from two different patients with subarachnoid hemorrhage secondary to rupture of the anterior communicating artery. All patients were admitted with a Glasgow Coma Score less than 7 and demonstrated elevated intracranial pressure requiring placement of an intraventricular shunt. All four CNS trauma patients demonstrated significant cTau7 labeled CSF proteins in the 30 kDa to 50 kDa range. In comparison, control patients demonstrated no cTau7 immunoreactivity (Fig. 1). Similar results were observed with CSF samples immunoblotted with cTau10 and cTau12 (data not shown). The developed Mabs resulted in more intense labeling of CNS trauma patient CSF than Tau-1. Split CSF samples with equivalent protein loads were blotted with Tau-1 (Fig. 1, left panel, lanes a and b) and cTau7 (Fig. 1, right panel, CNS trauma, lanes a and b) with cTau7 producing more intense labeling.

Characterization of cTau7, cTau8 and cTau12 labeled proteins

The experiments described below indicate that the 30 kDa to 50 kDa proteins recognized by antibodies cTau7, cTau8 and cTau12 are a cleaved form of the microtubule

binding protein tau. These data demonstrate that: 1) antibodies cTau7, cTau8 and cTau12 label recombinantly expressed tau, 2) that the 30 kDa to 50 kDa proteins recognized by cTau7, cTau8 and cTau12, similar to tau, contain a functionally intact microtubule binding domain, 3) antibodies cTau7, cTau8 and cTau12 label a 14.7 kDa CNBr digestion fragment comprised of pro²¹⁵ to met⁴¹⁹ of the tau primary sequence, 4) 30 kDa to 50 kDa cleaved tau proteins occur in both CSF from CNS trauma patients and *post mortem* brain and 5) epitope mapping studies suggest that CSF 30 kD to 50 kD proteins consist of the interior portion of the tau sequence from which the N- and C-terminal amino acids have been cleaved.

Recombinant tau immunoreactivity. Mabs cTau7, cTau8 and cTau12 labeled recombinant tau expressed in *E. coli* (Fig. 2). Recombinant tau containing either three repeats or four repeats was expressed in BL21(DE3) cells and isolated. Recombinant tau was highly purified as judged by Commassie Blue stained SDS-PAGE (Fig. 2, Gel). Both tau isoforms demonstrated immunoreactivity with tau polyclonal antibody BYA and at higher protein loads Mabs cTau12 (Fig. 2), cTau7 and cTau8 (data not shown).

Affinity and microtubule purification of tau. CSF from CNS trauma patients or a preparation of heat-stable brain proteins was affinity purified with Mabs cTau7, -8 and -12 (Fig. 3). Affinity purification from either source revealed a band of 30 kDa to 50 kDa tau proteins on immunoblotting or on Commassie Blue stained SDS-PAGE (Fig. 3, Affinity Purified). These data suggest that 30 kDa to 50 kDa tau proteins are cleaved in brain and then gain access to CSF.

Similar to tau, CSF and brain 30 kDa to 50 kDa proteins recognized by Mabs cTau7, cTau8 and cTau12 bind microtubules. Tubulin was purified from brain and polymerized into microtubules with taxol. Endogenous microtubule binding proteins were dissociated from the

polymerized microtubules with all detectable endogenous MAPs removed by the third salt extraction (Fig. 3, lane a). Microtubules were incubated with CSF 30 kDa to 50 kDa cleaved-tau proteins (Fig. 3, lane b) and microtubules washed several times to remove all proteins not tightly bound (Fig. 3, lanes c and d). Microtubule binding proteins were then salt extracted
5 from the washed microtubule preparation revealing a cTau7-labeled ladder of 30 kDa to 50 kDa proteins (Fig. 5, lane e). Similar results were obtained with Mabs cTau8 and cTau12 (data not shown). No labeling of MAP2 or neurofilaments with Mabs cTau7, 8 and 9 was observed in the microtubule purified brain preparation. The presence of 280 kDa MAP2 in this preparation was confirmed by immunoblots with Mab AP-14 (Kalcheva et al., 1994) which
10 labels both MAP2a and MAP2b while the presence of 200 kDa neurofilament-H and 155 kDa neurofilament-M was confirmed with Mabs NE-14 (Gotow and Tanaka, 1994) and BF-10 (Anderton et al., 1982), respectively (data not show).

Epitope mapping. cTau7, - 8 and -12 immunoblots of CNBr digested CSF or brain 30 kDa to 50 kDa tau proteins indicated that all three Mabs labeled the same CNBr fragment (Fig.
15 4). This fragment could also be labeled with the Mab PHF-1 which recognizes phosphorylated ser³⁹⁶ of tau (Otvos et al, 1994; Zemlan et al., 1996). The PHF-1 data identify the cTau7, cTau8 and cTau12 labeled fragment as the largest CNBr digestion product that is comprised of tau sequences pro²⁵¹ to met⁴¹⁹. These data indicate that both CSF and brain cleaved tau proteins demonstrate similar CNBr digestion products suggesting that the labeled CSF and brain
20 proteins are structurally similar.

Tau antibody BYA raised against full length tau labeled a band of 48 kDa to 68 kDa proteins in heat-stable extracts of *post mortem* brain (Fig. 5). In comparison, antibodies cTau7, -8 and -12 labeled a band of 30 kDa to 50 kDa proteins indicating that these cTau

Mabs labeled proteins demonstrating an greater gel mobility than intact tau. Further, these data indicate that Mabs cTau7, cTau8 and cTau12 have limited affinity for intact, full length tau. Mab PHF-1 also selectively labeled a band of 30 kDa to 50 kDa proteins indicating that these proteins differ from intact tau both by cleavage and phosphorylation at ser³⁹⁶ (Fig. 5, PHF1). Mab Tau-1 labeled proteins spanning the 30 kDa to 68 kDa region indicating that ser¹⁹⁹ is poorly phosphorylated in both full length and cleaved tau (Fig. 5, Tau1). In comparison, the C-terminal antibody Alz5 and the N-terminal antibody Alz50 labeled only full length tau suggesting that these epitopes are not present in cleaved tau.

To determine whether tau cleavage occurred in CSF, 5 µg of recombinant tau was added to 1 ml of CSF and incubated overnight at 37°C. CSF aliquots were run on SDS-PAGE and blotted with BYA which indicted the presence of intact, non-cleaved tau in all cases.

Prior to sandwich ELISA development, it was important to determine whether cTau7, cTau 8 and cTau12 epitopes were independent and whether the epitopes were phosphorylated. ELISA competition studies between cTau7, cTau 8 and cTau12 resulted in non-competitive binding to CSF tau proteins (data not shown). Also, Mabs cTau7, cTau 8 and cTau 12 demonstrated phosphorylation independent binding to 30 kDa to 50 kDa cleaved tau proteins. For example, BAP treatment produced a 5 fold increase in the binding of Mab SMI33 which recognizes non-phosphorylated ser²³⁵ of tau (BAP: 1.88, no BAP: 0.35, mean O.D.) while no effect of BAP treatment on cTau7 (BAP: 1.75, no BAP 1.51), cTau8 (BAP: 1.99, no BAP: 1.61) or cTau12 (BAP:1.72, no BAP: 1.53) binding was observed.

Cleaved tau sandwich ELISA and axonal degeneration

A sensitive ELISA using catalyzed reporter deposition for signal enhancement was

developed using Mab cTau12 for antigen capture, HRP-conjugated Mabs cTau7 and cTau8 for detection, and biotin-tyramine as the reporter. The sensitivity of this ELISA for affinity purified CSF tau was about 0.030 ng to 0.040 ng per well (Fig. 6).

This ELISA was employed to measure CSF cleaved tau levels in patients undergoing active axonal degeneration. CSF was obtained from 131 patients. Samples were collected from patients with axonal degeneration resulting from acute CNS injury and three control groups free of axonal degeneration (demyelinating disease, neurologic controls and normal controls). The CNS injury group consisted of patients with acute severe brain injury resulting from trauma or intracranial aneurysmal rupture. The demyelinating disease group consisted exclusively of patients with multiple sclerosis (N=41). The neurologic control group consisted of patients with migraine or vascular headache (N=9), dementia not associated with a neurodegenerative disease (N=11), myotonic disorder (N=4), seizure disorder (N=3), Guillian-Barre syndrome (N=2), hydrocephalus (N=2), seizure disorder (N=2), transverse myelitis (N=2) or other neurologic disorder (N=3). The normal control group were individuals with no known neurologic disease. They consisted of family members of patients with a neurologic disease (N=10), patients with a psychiatric disorder (N=13) or patients hospitalized for a non-neurologic disorder (N=18).

CSF cleaved tau levels were significantly elevated in patients with axonal degeneration following CNS injury (Table 1). CSF cleaved tau levels were elevated over 10,000 fold in CNS injury patients when compared to patients without axonal degeneration, i.e. a demyelinating disease ($p < 0.001$), or neurologic controls ($p < 0.001$) or normal controls ($p < 0.001$). Further, no overlap between CNS injury CSF cleaved tau levels and patients in any control group were observed. Only two of the 41 patients in the demyelinating disease

group demonstrated detectable tau levels and both values were significantly lower than those observed in the CNS injury group. Similarly, CSF cleaved tau levels were not detectable in most control patients. Among neurologic controls only two of 38 patients had detectable CSF tau levels. These were patients with Guillian Barre syndrome (0.464 ng/ml) or cerebral ischemia (0.433 ng/ml). Only one of 41 normal control patients demonstrated detectable tau levels. This was a cardiac transplant patient (0.927 ng/ml).

Conclusion

In the present disclosure, a novel cleaved form of tau is described in CSF. This truncated form of tau was reactive with the three Mabs developed in the present study and tau antibodies raised in other laboratories (Figs. 1 and 5). The present data indicate that only cleaved tau and not full length 48 kDa to 68 kDa tau proteins are present in CSF (Cleveland et al., 1977; Couchie and Nunez, 1985). Consistent with these data, differential CSF hybridoma screening identified three Mabs that demonstrated high affinity for the cleaved form of tau found in CSF but limited affinity for intact, full length tau (Fig. 5). For example, Mabs cTau7, cTau8 and cTau12 demonstrated selective labeling of cleaved tau when equivalent protein loads of cleaved tau and intact were examined by Western blot (Fig. 5). Similarly, thirty fold greater protein loads of full length recombinant tau were required to demonstrate labeling with cTau7, cTau8 and cTau12 than with antibodies raised against full length tau (Fig. 2).

The selectivity of Mabs cTau7, cTau8 and cTau12 for cleaved tau is best explained by a conformational state assumed by tau following cleavage rather than differences in primary sequence or phosphorylation. Previous research demonstrates that Mabs can specifically

recognize tau secondary structure. Several tau Mabs including Alz50, Tau-2 and MC1 recognize a specific tau conformation rather than tau primary structure (Carmel et al., 1996; Vincent et al., 1996). For example, using tau deletion mutants Carmel et al. (1996) demonstrated that Alz50 recognized a conformation of tau where the N-terminus is in close
5 association with the microtubule binding domain. The K_d of Alz50 for the native tau protein in this preferred conformation was over 70 fold higher than for the protein without the preferred secondary structure. Similarly, Mabs cTau7, cTau8 and cTau12 appear to recognize a secondary structure that tau assumes after cleavage. The present CNBr, epitope mapping and microtubule binding data indicate that 30 kDa to 50 kDa CSF cleaved tau proteins are
10 comprised of the interior portion of the tau primary sequence including a functionally intact microtubule binding domain (Figs 3, 4 and 5).

The present data indicate that CSF levels of cleaved-tau reflect the extent of CNS axonal degeneration. CSF cleaved-tau levels were elevated 10,000 fold over control levels in patients hospitalized for CNS injury. Further, there was no overlap of CSF cleaved tau levels
15 between acute CNS injury patients and controls. The sequelae of CNS injury is characterized by extended parenchymal atrophy and axonal degeneration (Adams et al., 1989). Microtubule-associated protein tau is a neuron-specific cytoskeletal protein localized in the axonal compartment. The present data indicate that axonal degeneration is associated with significant release of cleaved tau into CSF.

Table 1. CSF cleaved tau levels and demographic data for patients with CNS injury and three control groups (demyelinating disease, neurologic and normal controls).

Diseases	CSF cleaved-Tau (ng/ml)			Age		N
	mean	range	SD	mean	SD	
CNS Injury ^{a,b,c}	396.96	2.5-2,361	741.58	47.4	15.2	11
Demyelinating	0.005	0-0.21	0.03	40.3	10.3	41
Neurologic Controls	0.024	0-0.50	0.10	52.2	17.5	38
Normal Controls	0.023	0-0.93	0.15	53.8	16.0	41

Analysis of variance of CSF tau levels for four patients groups, $p < 0.001$.

^a CSF tau levels are significantly different from neurologic control group, $p < 0.001$.

^b CSF tau levels are significantly different from normal control group, $p < 0.001$.

^c CSF tau levels significantly elevated from demyelinating disease group, $p < 0.001$.

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What Is Claimed Is:

1. A monoclonal antibody raised against tau protein(s) and having been screened against human cerebrospinal fluid, said monoclonal antibody being in a substantially isolated form.
2. A monoclonal antibody raised against a tau protein fragment(s), and having been screened against human cerebrospinal fluid, said monoclonal antibody being in a substantially purified form, and said tau protein fragment(s) including the amino acids from serine¹⁹⁹ to serine³⁹⁶ of tau protein.
3. A monoclonal antibody according to claim 2, raised against a tau protein fragment(s), and having been screened against human cerebrospinal fluid, said monoclonal antibody being in a substantially purified form, and said tau protein fragment(s) including the sequence from serine¹⁹⁹ to serine³⁹⁶ of tau protein, and lacking the native N-terminal and C-terminal amino acids.
4. A protein fragment of an isoform of tau protein, said protein fragment having an apparent molecular weight less than 50 kDa, and being in a substantially isolated form.
5. A protein fragment of an isoform of tau protein according to claim 4 wherein said protein fragment is comprised of the sequence including amino acids serine¹⁹⁹ to serine³⁹⁶ of tau proteins.
6. A protein fragment of an isoform of tau protein according to claim 4 wherein said protein fragment is comprised of the sequence including amino acids serine¹⁹⁹ to serine³⁹⁶ of tau proteins, and lacking the native N-terminal and C-terminal amino acids.
7. A protein fragment of an isoform of tau protein according to claim 4 wherein said protein fragment possesses an apparent molecular weight of about 30 kDa to about 50 kDa, and being in a substantially isolated form.

8. A protein fragment of an isoform of tau protein according to claim 7 wherein said protein fragment is comprised of the sequence including amino acids serine¹⁹⁹ to serine³⁹⁶ of tau proteins.
9. A protein fragment of an isoform of tau protein according to claim 7 wherein said protein fragment is comprised of the sequence including amino acids serine¹⁹⁹ to serine³⁹⁶ of tau proteins, and lacking the native N-terminal and C-terminal amino acids.
10. A protein fragment of the light neurofilament subunit, said protein fragment having an apparent molecular weight less than 68 kDa, and being in a substantially isolated form.
11. A protein fragment of the medium-sized neurofilament subunit, said protein fragment having an apparent molecular weight less than 160 kDa, and being in a substantially isolated form.
12. A protein fragment of the heavy neurofilament subunit, said protein fragment having an apparent molecular weight less than 200 kDa, and being in a substantially isolated form.
13. A protein fragment of neurofilament66, said protein fragment having an apparent molecular weight less than 66 kDa, and being in a substantially isolated form.

Method For Determining Axonal Damage

14. A method of determining axonal damage in the human central nervous system, said method comprising the steps:
 - (a) obtaining a sample of cerebrospinal fluid from said human central nervous system;
 - (b) treating said sample of cerebrospinal fluid with at least one monoclonal antibody, said at least one monoclonal antibody having been raised against an axonally-derived protein; and
 - (c) detecting the presence of said axonally-derived protein bound to said at least one monoclonal antibody.

15. A method according to claim 14 additionally comprising the step of comparing the amount of said axonally-derived protein bound to said at least one monoclonal antibody in step (c) to control samples selected from the group representing a normal undamaged axon state and those representing an axonal damage state.
16. A method according to claim 14 wherein said axonally derived protein is at least one isoform of tau protein.
17. A method according to claim 16 wherein said at least one isoform of said tau protein is a fragment of said tau protein demonstrating an apparent molecular weight less than 50 kDa.
18. A method according to claim 17 wherein said at least one tau protein fragment demonstrating an apparent molecular weight in the range of about 30 kDa to about 50 kDa.
19. A method according to claim 18 wherein said at least one tau protein fragment comprises the sequence from serine¹⁹⁹ to serine³⁹⁶ of tau protein.
20. A method according to claim 19 wherein said at least one tau protein fragment lacks the native N-terminal and C-terminal amino acids.
21. A method according to claim 14 wherein said axonally-derived protein is at least one neurofilament protein from the class of neurofilament proteins consisting of the light neurofilament subunit, the medium-sized neurofilament subunit, the heavy neurofilament subunit and/or neurofilament66.
22. A method according to claim 21 wherein said neurofilament protein is a fragment of the full length neurofilament protein found in human cerebrospinal fluid.
23. A method according to claim 14 wherein said presence of said axonally-derived protein bound to said at least one monoclonal antibody is detected through gel electrophoresis.
24. A method according to claim 23 wherein said axonally-derived protein bound to said at

least one monoclonal antibody is a fragment of tau protein which is detected through gel electrophoresis and which gives rise to an electrophoresis gel demonstrating multiple protein bands with apparent molecular weights less than 50 kDa.

25. A method according to claim 24 wherein said axonally-derived protein bound to said at least one monoclonal antibody is a fragment of tau protein which is detected through gel electrophoresis and which gives rise to an electrophoresis gel demonstrating multiple protein bands with apparent molecular weights from about 30 to 50 kDa.

26. A method according to claim 14 further comprising the measurement of said axonally derived proteins in said cerebrospinal fluid by an ELISA technique.

27. The method of claim 26 wherein the ELISA employs monoclonal antibodies recognizing tau protein present in human cerebrospinal fluid.

28. The method of claim 26 wherein the monoclonal antibodies comprise Mabs cTau7, cTau8 and/or cTau12.

29. The method of claim 26 wherein said ELISA is a tau sandwich ELISA.

30. A method according to claim 14 wherein said cerebrospinal fluid is collected from a patient with a neurological disease selected from the group consisting of traumatic central nervous system injury, central nervous system tumor, neurodegenerative diseases of the central nervous system including Alzheimer's Disease, spinal cord injury or cerebral vascular accident.

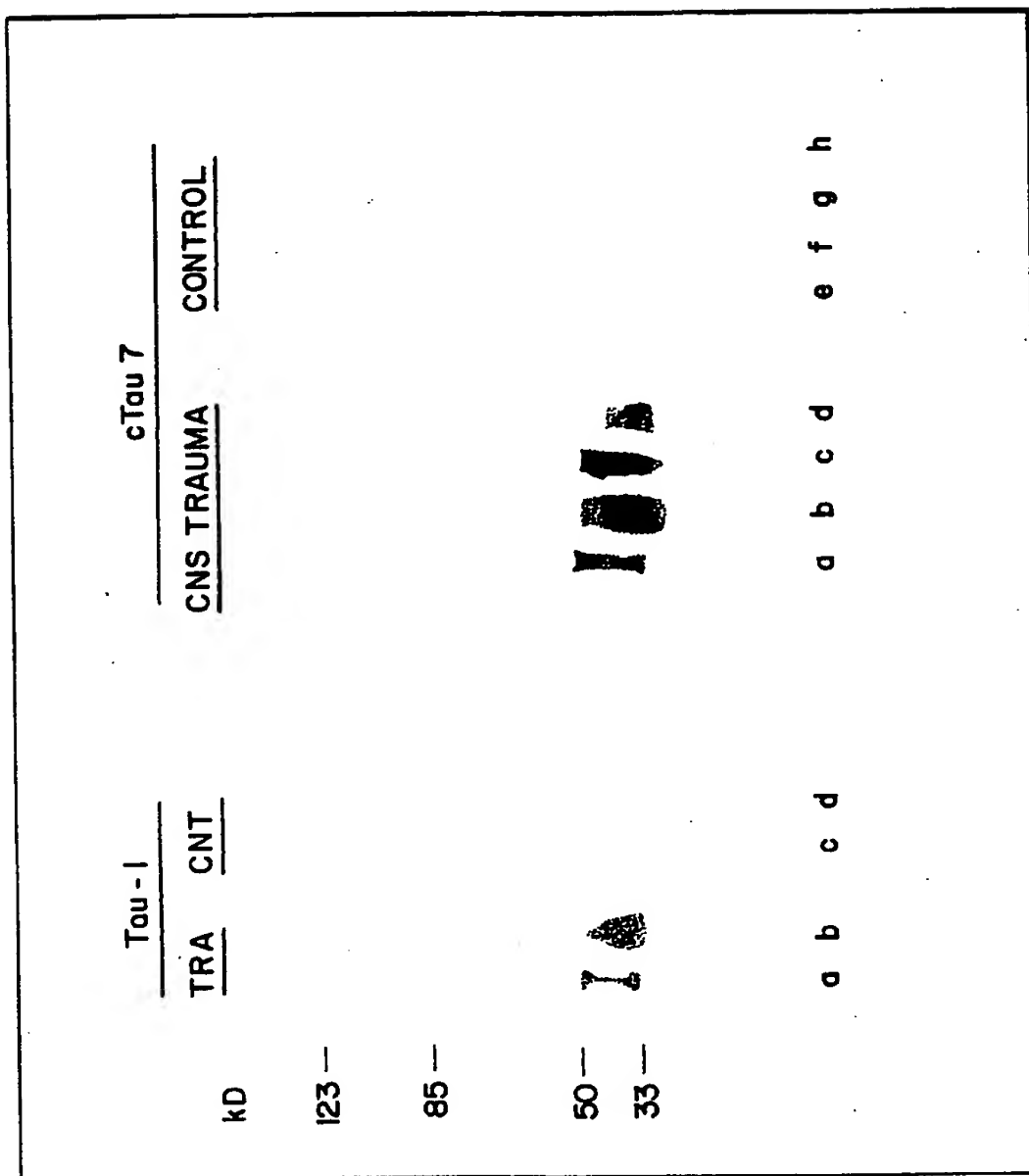


Fig. 1

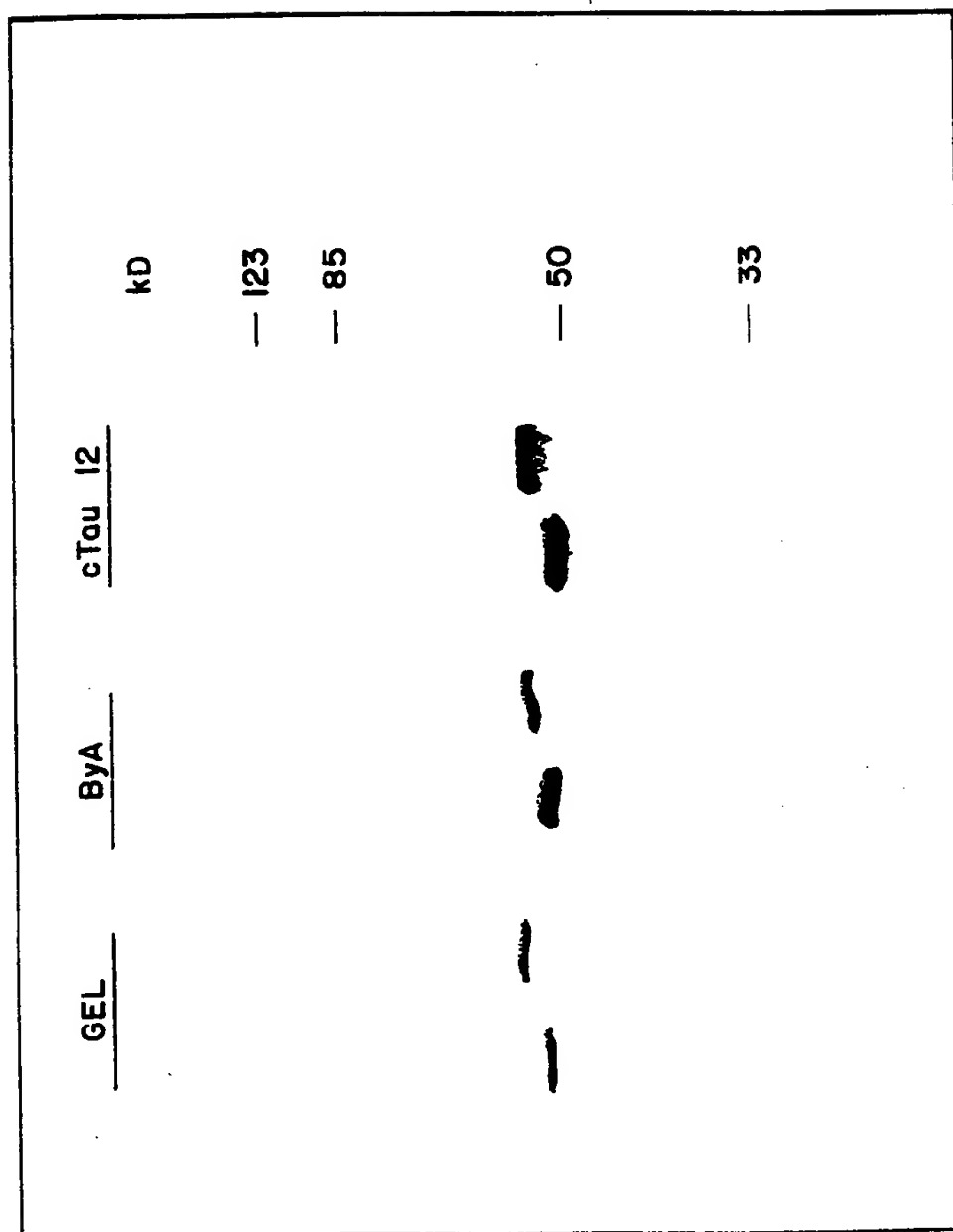


Fig. 2

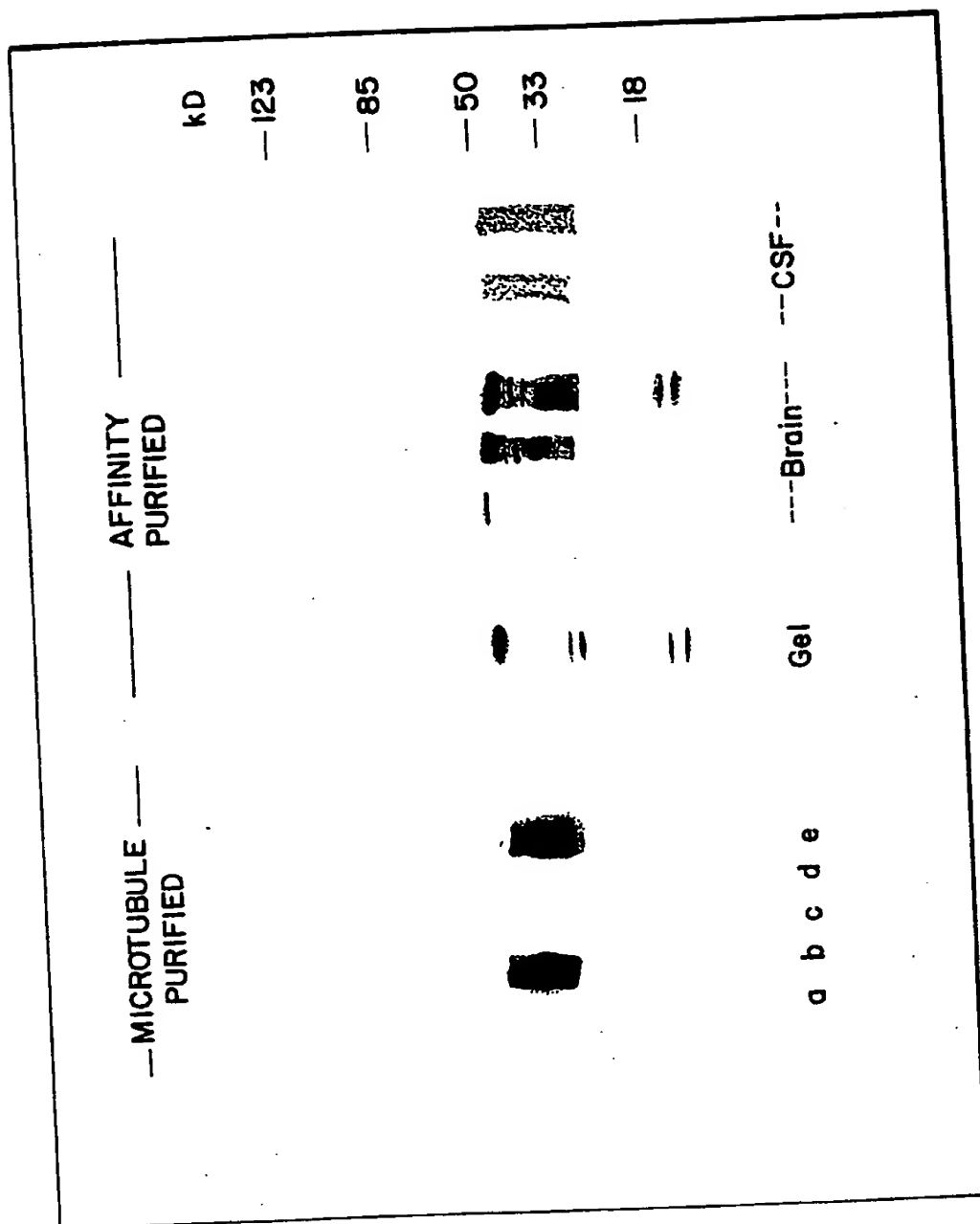


Fig. 3

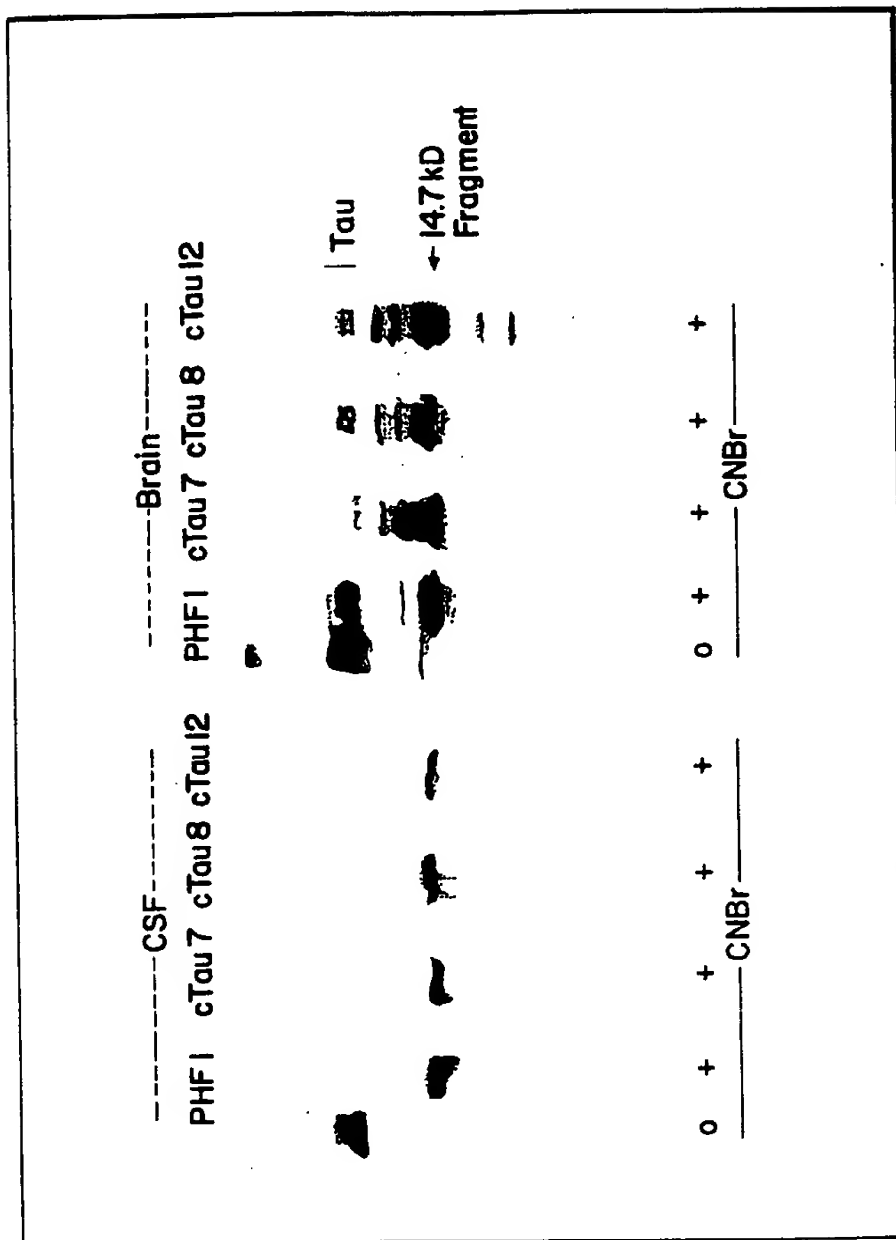


Fig. 4

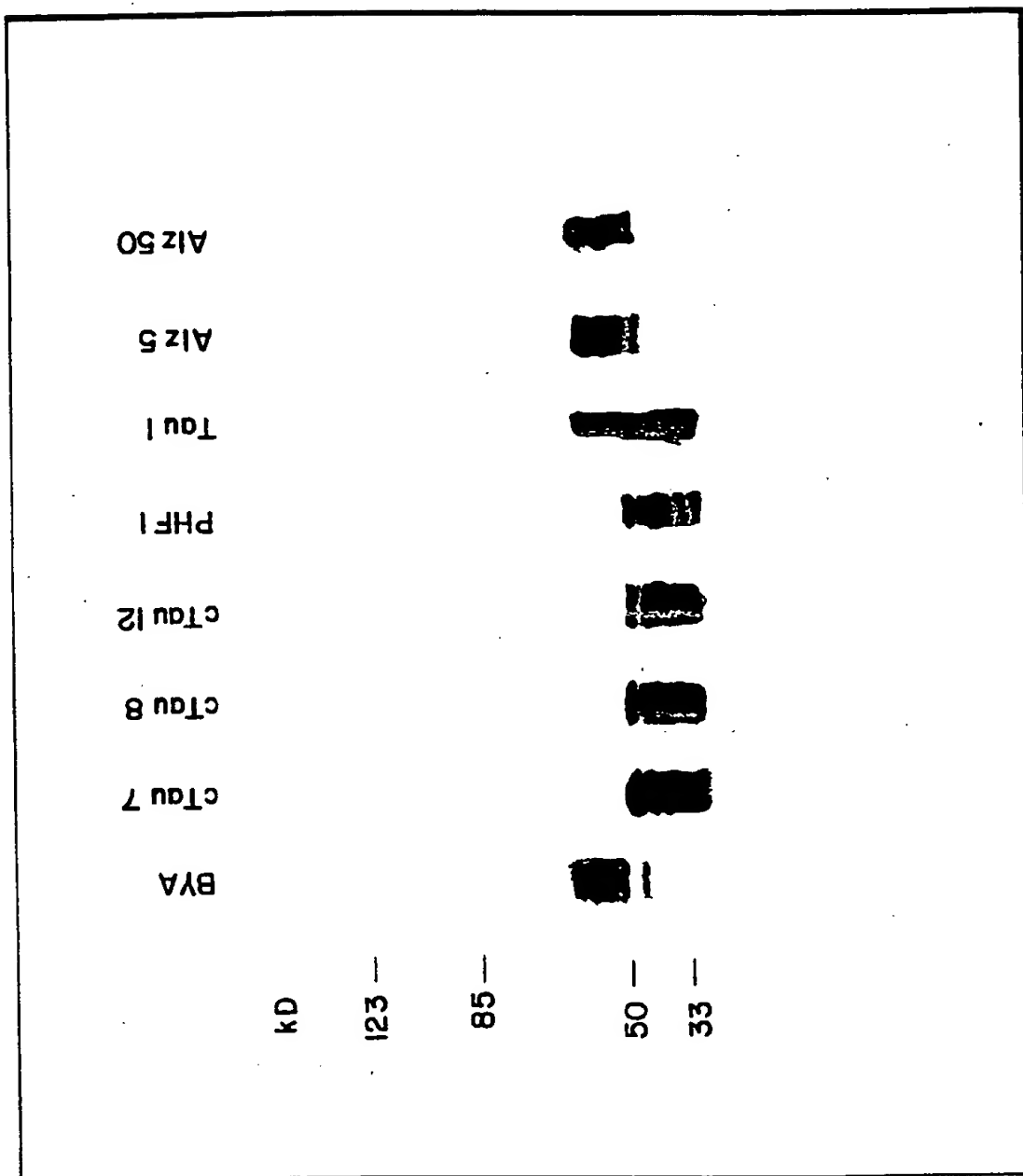
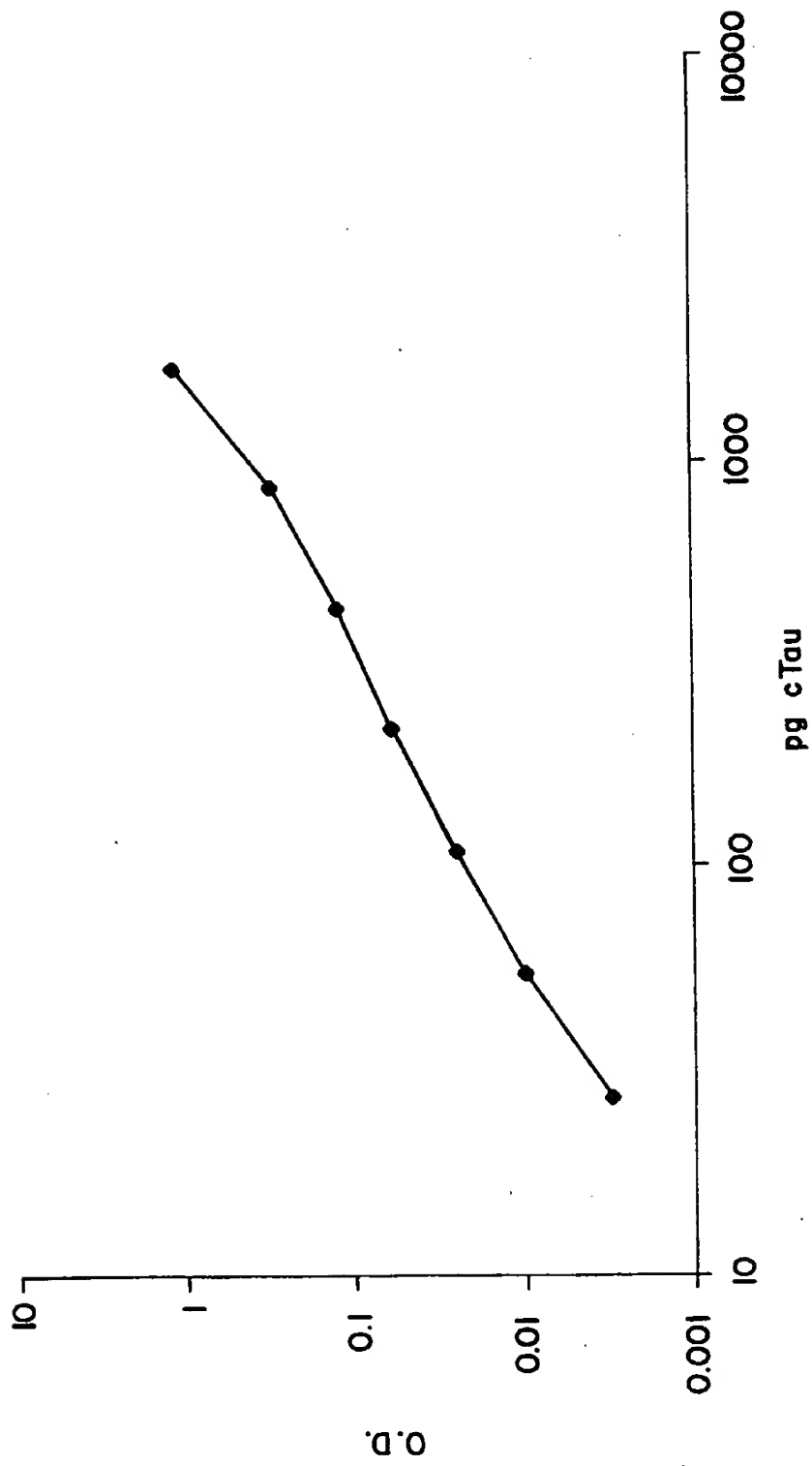


Fig. 5

*Fig. 6*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04955

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53; C07K 16/00

US CL :435/7.1; 530/350, 387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1; 530/350, 387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/03369 A1 (VOORHEIS) 18 February 1993, see entire document.	1-30
X	WO 93/08302 A1 (N.V. INNOGENETICS S.A.) 29 April 1993, see entire document.	1-30
X	WO 93/11231 A1 (MAXPLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN E.V.) 10 June 1993, see entire document.	1-30
X	WO 94/13795 A1 (N.V. INNOGENETICS S.A.) 23 June 1994, see entire document.	1-30
X	WO 95/17429 A1 (INNOGENETICS N.V.) 29 June 1995, see entire document.	1-30
X	WO 96/04309 A1 (INNOGENETICS N.V.) 15 February 1996, see	1-30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 MAY 1999

Date of mailing of the international search report

11 JUN 1999

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/04955

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DERWENT WPI, MEDLINE, BIOSYS, JAPIO, EMBASE.

Search terms: antibod?, immunoglob?, monoclonal, tau, neurofilament(s), Alzheimer's disease, isolate, cerebrospinal, immunoassay, tau fragment(s), axon, axonal

